Chapter 1

History of Plant Cell Culture

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INTRODUCTION

Plant cell/tissue culture, also referred to as *in vitro,* axenic, or sterile culture, is an important tool in both basic and applied studies as well as in commercial application (see Thorpe, 1990, 2007 and Stasolla & Thorpe 2011). Although Street (1977) has recommended a more restricted use of the term, plant tissue culture is generally used for the aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions *in vitro.* Perhaps the earliest step toward plant tissue culture was made by Henri-Louis Duhumel du Monceau in 1756, who, during his pioneering studies on wound-healing in plants, observed callus formation (Gautheret, 1985). Extensive microscopic studies led to the independent and almost simultaneous development of the cell theory by Schleiden (1838) and Schwann (1839). This theory holds that the cell is the unit of structure and function in an organism and therefore capable of autonomy. This idea was tested by several researchers, but the work of Vöchting (1878) on callus formation and on the limits to divisibility of plant segments was perhaps the most important. He showed that the upper part of a stem segment always produced buds and the lower end callus or

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roots independent of the size until a very thin segment was reached. He demonstrated polar development and recognized that it was a function of the cells and their location relative to the cut ends.

The theoretical basis for plant tissue culture was proposed by Gottlieb Haberlandt in his address to the German Academy of Science in 1902 on his experiments on the culture of single cells (Haberlandt, 1902). He opined that to "my knowledge, no systematically organized attempts to culture isolated vegetative cells from higher plants have been made. Yet the results of such culture experiments should give some interesting insight to the properties and potentialities which the cell as an elementary organism possesses. Moreover, it would provide information about the inter-relationships and complementary influences to which cells within a multicellular whole organism are exposed" (from the English translation by Krikorian & Berquam, 1969). He experimented with isolated photosynthetic leaf cells and other functionally differentiated cells and was unsuccessful, but nevertheless he predicted that "one could successfully cultivate artificial embryos from vegetative cells." He thus clearly established the concept of totipotency, and further indicated that "the technique of cultivating isolated plant cells in nutrient solution permits the investigation of important problems from a new experimental approach." On the basis of that 1902 address and his pioneering experimentation before and later, Haberlandt is justifiably recognized as the father of plant tissue culture. Greater detail on the early pioneering events in plant tissue culture can be found in White (1963), Bhojwani and Razdan (1983), and Gautheret (1985).

THE EARLY YEARS

Using a different approach Kotte (1922), a student of Haberlandt, and Robbins (1922) succeeded in culturing isolated root tips. This approach, of using explants with meristematic cells, led to the successful and indefinite culture of tomato root tips by White (1934a). Further studies allowed for root culture on a completely defined medium. Such root cultures were used initially for viral studies and later as a major tool for physiological studies (Street, 1969). Success was also achieved with bud cultures by Loo (1945) and Ball (1946).

Embryo culture also had its beginning early in the nineteenth century, when Hannig in 1904 successfully cultured cruciferous embryos and Brown in 1906 barley embryos (Monnier, 1995). This was followed by the successful rescue of embryos from nonviable seeds of a cross between *Linum perenne* × *L. austriacum* (Laibach, 1929). Tukey (1934) was able to allow for full embryo development in some early-ripening species of fruit trees, thus providing one of the earliest applications of *in vitro* culture. The phenomenon of precocious germination was also encountered (LaRue, 1936).

The first true plant tissue cultures were obtained by Gautheret (1934, 1935) from cambial tissue of *Acer pseudoplatanus.* He also obtained success with similar explants of *Ulmus campestre*, *Robinia pseudoacacia*, and *Salix capraea*

using agar-solidified medium of Knop's solution, glucose, and cysteine hydrochloride. Later, the availability of indole acetic acid and the addition of B vitamins allowed for the more or less simultaneous demonstrations by Gautheret (1939) and Nobécourt (1939a) with carrot root tissues and White (1939a) with tumor tissue of a *Nicotiana glauca* × *N. langsdorffii* hybrid, which did not require auxin, that tissues could be continuously grown in culture and even made to differentiate roots and shoots (Nobécourt, 1939b; White, 1939b). However, all of the initial explants used by these pioneers included meristematic tissue. Nevertheless, these findings set the stage for the dramatic increase in the use of *in vitro* cultures in the subsequent decades.

THE ERA OF TECHNIQUES DEVELOPMENT

The 1940s, 1950s, and 1960s proved an exciting time for the development of new techniques and the improvement of those already available. The application of coconut water (often incorrectly stated as coconut milk) by Van Overbeek *et al.* (1941) allowed for the culture of young embryos and other recalcitrant tissues, including monocots. As well, callus cultures of numerous species, including a variety of woody and herbaceous dicots and gymnosperms as well as crown gall tissues, were established (see Gautheret, 1985). Also at this time, it was recognized that cells in culture underwent a variety of changes, including loss of sensitivity to applied auxin or habituation (Gautheret, 1942, 1955) as well as variability of meristems formed from callus (Gautheret, 1955; Nobécourt, 1955). Nevertheless, it was during this period that most of the *in vitro* techniques used today were largely developed.

Studies by Skoog and his associates showed that the addition of adenine and high levels of phosphate allowed nonmeristematic pith tissues to be cultured and to produce shoots and roots, but only in the presence of vascular tissue (Skoog & Tsui, 1948). Further studies using nucleic acids led to the discovery of the first cytokinin (kinetin) as the breakdown product of herring sperm DNA (Miller *et al.*, 1955). The availability of kinetin further increased the number of species that could be cultured indefinitely, but perhaps most importantly, led to the recognition that the exogenous balance of auxin and kinetin in the medium influenced the morphogenic fate of tobacco callus (Skoog & Miller, 1957). A relative high level of auxin to kinetin favored rooting, the reverse led to shoot formation, and intermediate levels to the proliferation of callus or wound parenchyma tissue. This morphogenic model has been shown to operate in numerous species (Evans *et al.,* 1981). Native cytokinins were subsequently discovered in several tissues, including coconut water (Letham, 1974). In addition to the formation of unipolar shoot buds and roots, the formation of bipolar somatic embryos (carrot) were first reported independently by Reinert (1958, 1959) and Steward *et al.* (1958).

The culture of single cells (and small cell clumps) was achieved by shaking callus cultures of *Tagetes erecta* and tobacco and subsequently placing them on

filter paper resting on well-established callus, giving rise to the so-called nurse culture (Muir *et al.*, 1954, 1958). Later, single cells could be grown in medium in which tissues had already been grown, i.e., conditioned medium (Jones *et al.*, 1960). As well, Bergmann (1959) incorporated single cells in a 1-mm layer of solidified medium where some cell colonies were formed. This technique is widely used for cloning cells and in protoplast culture (Bhojwani & Razdan, 1983). Kohlenbach (1959) finally succeeded in the culture of mechanically isolated mature differentiated mesophyll cells of *Macleaya cordata* and later induced somatic embryos from callus (Kohlenbach, 1966). The first large-scale culture of plant cells was reported by Tulecke and Nickell (1959), who grew cell suspensions of *Ginkgo*, holly, *Lolium*, and rose in simple sparged 20-liter carboys. Utilizing coconut water as an additive to fresh medium, instead of using conditioned medium, Vasil and Hildebrandt (1965) finally realized Haberlandt's dream of producing a whole plant (tobacco) from a single cell, thus demonstrating the totipotency of plant cells.

The earliest nutrient media used for growing plant tissues *in vitro* were based on the nutrient formulations for whole plants, for which they were many (White, 1963); but Knop's solution and that of Uspenski and Uspenskia were used the most and provided less than 200 mg/liter of total salts. Heller (1953), based on studies with carrot and Virginia creeper tissues, increased the concentration of salts twofold, and Nitsch and Nitsch (1956) further increased the salt concentration to ca 4 g/liter, based on their work with Jerusalem artichoke. However, these changes did not provide optimum growth for tissues, and complex addenda, such as yeast extract, protein hydrolysates, and coconut water, were frequently required. In a different approach based on an examination of the ash of tobacco callus, Murashige and Skoog (1962) developed a new medium. The concentration of some salts were 25 times that of Knop's solution. In particular, the level of NO_3^- and NH_4^+ were very high and the array of micronutrients were increased. This formulation allowed for a further increase in the number of plant species that could be cultured, many of them using only a defined medium consisting of macro- and micronutrients, a carbon source, reduced nitrogen, B vitamins, and growth regulators (Gamborg *et al.*, 1976).

Ball (1946) successfully produced plantlets by culturing shoot tips with a couple of primordia of *Lupinus* and *Tropaeolum*, but the importance of this finding was not recognized until Morel (1960), using this approach to obtain virus-free orchids, realized its potential for clonal propagation. The potential was rapidly exploited, particularly with ornamentals (Murashige, 1974). Early studies by White (1934b) showed that cultured root tips were free of viruses. Later Limmaset and Cornuet (1949) observed that the virus titer in the shoot meristem was very low. This was confirmed when virus-free *Dahlia* plants were obtained from infected plants by culturing their shoot tips (Morel & Martin, 1952). Virus elimination was possible because vascular tissue, in which the viruses move, do not extend into the root or shoot apex. The method was further refined by Quack (1961) and is now routinely used.

Techniques for *in vitro* culture of floral and seed parts were developed during this period. The first attempt at ovary culture was by LaRue (1942), who obtained limited growth of ovaries accompanied by rooting of pedicels in several species. Compared to studies with embryos, successful ovule culture is very limited. Studies with both ovaries and ovules have been geared mainly to an understanding of factors regulating embryo and fruit development (Rangan, 1982). The first continuously growing tissue cultures from an endosperm were from immature maize (LaRue, 1949); later, plantlet regeneration via organogenesis was achieved in *Exocarpus cupressiformis* (Johri & Bhojwani, 1965).

In vitro pollination and fertilization was pioneered by Kanta *et al.* (1962) using *Papaver somniferum.* The approach involves culturing excised ovules and pollen grains together in the same medium and has been used to produce interspecific and intergeneric hybrids (Zenkteler *et al.*, 1975). Earlier, Tuleke (1953) obtained cell colonies from *Ginkgo* pollen grains in culture, and Yamada *et al.* (1963) obtained haploid callus from whole anthers of *Tradescantia reflexa.* However, it was the finding of Guha and Maheshwari (1964, 1966) that haploid plants could be obtained from cultured anthers of *Datura innoxia* that opened the new area of androgenesis. Haploid plants of tobacco were also obtained by Bourgin and Nitsch (1967), thus confirming the totipotency of pollen grains.

Plant protoplasts or cells without cell walls were first mechanically isolated from plasmolyzed tissues well over 100 years ago by Klercker in 1892, and the first fusion was achieved by Küster in 1909 (Gautheret, 1985). Nevertheless, this remained an unexplored technology until the use of a fungal cellulase by Cocking (1960) ushered in a new era. The commercial availability of cell-wall-degrading enzymes led to their wide use and the development of protoplast technology in the 1970s. The first demonstration of the totipotency of protoplasts was by Takebe *et al.* (1971), who obtained tobacco plants from mesophyll protoplasts. This was followed by the regeneration of the first interspecific hybrid plants (*Nicotiana glauca* × *Nicotiana langsdorffii*) by Carlson *et al.* (1972).

Braun (1941) showed that *Agrobacterium tumefaciens* could induce tumors in sunflower, not only at the inoculated sites, but at distant points. These secondary tumors were free of bacteria and their cells could be cultured without auxin (Braun & White, 1943). Further experiments showed that crown gall tissues, free of bacteria, contained a tumor-inducing principle (TIP), which was probably a macromolecule (Braun, 1950). The nature of the TIP was worked out in the 1970s (Zaenen *et al.*, 1974), but Braun's work served as the foundation for *Agrobacterium*-based transformation. It should also be noted that the finding by Ledoux (1965) that plant cells could take up and integrate DNA remained controversial for over a decade.

THE RECENT PAST

Based on the availability of the various *in vitro* techniques discussed above, it is not surprising that, starting in the mid-1960s, there was a dramatic increase in

their application to various problems in basic biology, agriculture, horticulture, and forestry through the 1970s and 1980s. These applications can be divided conveniently into five broad areas, namely: (a) cell behavior, (b) plant modification and improvement, (c) pathogen-free plants and germplasm storage, (d) clonal propagation, and (e) product formation (Thorpe, 1990). Detailed information on the approaches used can be gleaned from Bhojwani and Razdan (1983), Vasil (1984), Vasil and Thorpe (1994), and Stasolla and Thorpe (2011), among several sources.

Cell Behavior

Included under this heading are studies dealing with cytology, nutrition, and primary and secondary metabolism as well as morphogenesis and pathology of cultured tissues (Thorpe, 1990). Studies on the structure and physiology of quiescent cells in explants, changes in cell structure associated with the induction of division in these explants, and the characteristics of developing callus and cultured cells and protoplasts have been carried out using light and electron microscopy (Yeoman & Street, 1977; Lindsey & Yeoman, 1985; Fowke 1986, 1987). Nuclear cytology studies have shown that endoreduplication, endomitosis, and nuclear fragmentation are common features of cultured cells (D'Amato, 1978; Nagl *et al.*, 1985).

Nutrition was the earliest aspect of plant tissue culture investigated, as indicated earlier. Progress has been made in the culture of photoautotrophic cells (Yamada *et al.*, 1978; Hüsemann, 1985). *In vitro* cultures, particularly cell suspensions, have become very useful in the study of both primary and secondary metabolism (Neumann *et al.*, 1985). In addition to providing protoplasts from which intact and viable organelles were obtained for study (e.g., vacuoles; Leonard & Rayder, 1985), cell suspensions have been used to study the regulation of inorganic nitrogen and sulfur assimilation (Filner, 1978), carbohydrate metabolism (Fowler, 1978), and photosynthetic carbon metabolism (Bender *et al.*, 1985; Herzbeck & Hüsemann, 1985), thus clearly showing the usefulness of cell cultures for elucidating pathway activity. Most of the work on secondary metabolism was related to the potential of cultured cells to form commercial products, but has also yielded basic biochemical information (Constabel & Vasil, 1987, 1988).

Morphogenesis or the origin of form is an area of research with which tissue culture has long been associated and one to which tissue culture has made significant contributions in terms of both fundamental knowledge and application (Thorpe, 1990). Xylogenesis or tracheary element formation has been used to study cytodifferentiation (Roberts, 1976; Phillips, 1980; Fukuda & Komamine, 1985). In particular the optimization of the *Zinnia* mesophyll single-cell system has dramatically improved our knowledge of this process. The classic findings of Skoog and Miller (1957) on the hormonal balance for organogenesis has continued to influence research on this topic, a concept supported more recently by

transformation of cells with appropriately modified *Agrobacterium* T-DNA (Schell *et al.*, 1982; Schell, 1987). However, it is clear from the literature that several additional factors, including other growth-active substances, interact with auxin and cytokinin to bring about *de novo* organogenesis (Thorpe, 1980). In addition to bulky explants, such as cotyledons, hypocotyls, and callus (Thorpe, 1980), thin (superficial) cell layers (Tran Thanh Van & Trinh, 1978; Tran Thanh Van, 1980) have been used in traditional morphogenic studies, as well as to produce *de novo* organs and plantlets in hundreds of plant species (Murashige, 1974, 1979). Furthermore, physiological and biochemical studies on organogenesis have been carried out (Thorpe, 1980; Brown & Thorpe, 1986; Thompson & Thorpe, 1990). The third area of morphogenesis, somatic embryogenesis, also developed in this period and by the early 1980s over 130 species were reported to form bipolar structures (Ammirato, 1983; Thorpe, 1988). Successful culture was achieved with cereals, grasses, legumes, and conifers, previously considered to be recalcitrant groups. The development of a single-cell-to-embryo system in carrot (Normura & Komamine, 1985) allows for an in-depth study of the process.

Cell cultures have continued to play an important role in the study of plant–microbe interaction, not only in tumorigenesis (Butcher, 1977), but also on the biochemistry of virus multiplication (Rottier, 1978), phytotoxin action (Earle, 1978), and disease resistance, particularly as affected by phytoalexins (Miller & Maxwell, 1983). Without doubt the most important studies in this area dealt with *Agrobacteria*, and, although aimed mainly at plant improvement (see below), provided good fundamental information (Schell, 1987).

Plant Modification and Improvement

During this period *in vitro* methods were used increasingly as an adjunct to traditional breeding methods for the modification and improvement of plants. The technique of controlled *in vitro* pollination on the stigma, placenta, or ovule has been used for the production of interspecific and intergeneric hybrids, overcoming sexual self-incompatibility, and the induction of haploid plants (Yeung *et al.*, 1981; Zenkteler, 1984). Embryo, ovary, and ovule cultures have been used in overcoming embryo inviability, monoploid production in barley, and seed dormancy and related problems (Raghavan, 1980; Yeung *et al.*, 1981). In particular, embryo rescue has played a most important role in producing interspecific and intergeneric hybrids (Collins & Grosser, 1984).

By the early 1980s, androgenesis had been reported in some 171 species, of which many were important crop plants (Hu & Zeng, 1984). Gynogenesis was reported in some 15 species, in some of which androgenesis was not successful (San & Gelebart, 1986). The value of these haploids was that they could be used to detect mutations and for recovery of unique recombinants, since there is no masking of recessive alleles. As well, the production of double-haploids allowed for hybrid production and their integration into breeding programs.

Cell cultures have also played an important role in plant modification and improvement, as they offer advantages for isolation of variants (Flick, 1983). Although tissue culture-produced variants have been known since the 1940s, e.g., habituation, it was only in the 1970s that attempts were made to utilize them for plant improvement. This somaclonal variation is dependent on the natural variation in a population of cells, either preexisting or culture induced, and is usually observed in regenerated plantlets (Larkin & Scowcroft, 1981). The variation may be genetic or epigenetic and is not simple in origin (Larkin *et al.*, 1985; Scowcroft *et al.*, 1987), but the changes in the regenerated plantlets have potential agricultural and horticultural significance. It has also been possible to produce a wide spectrum of mutant cells in culture (Jacobs *et al.*, 1987). These include cells showing biochemical differences and antibiotic-, herbicide-, and stress-resistance. In addition, auxotrophs, autotrophs, and those with altered developmental systems have been selected in culture; usually the application of the selective agent in the presence of a mutagen is required. However, in only a few cases has it been possible to regenerate plants with the desired traits, e.g., herbicide-resistant tobacco (Hughes, 1983) and methyl tryptophan-resistant *Datura innoxia* (Ranch *et al.*, 1983).

By 1985 nearly 100 species of angiosperms could be regenerated from protoplasts (Binding, 1986). The ability to fuse plant protoplasts by chemical (e.g., with PEG) and physical (e.g., electrofusion) means allowed for production of somatic hybrid plants, the major problem being the ability to regenerate plants from the hybrid cells (Evans *et al.*, 1984; Schieder & Kohn, 1986). Protoplast fusion has been used to produce unique nuclear-cytoplasmic combinations. In one such example, *Brassica campestris* chloroplasts coding for atrazine resistance (obtained from protoplasts) were transferred into *Brassica napus* protoplasts with *Raphanus sativus* cytoplasm (which confers cytoplasmic male sterility from its mitochondria). The selected plants contained *B. napus* nuclei, chloroplasts from *B. campestris,* and mitochondria from *R. sativus*; had the desired traits in a *B. napus* phenotype; and could be used for hybrid seed production (Chetrit *et al.*, 1985). Unfortunately, only a few such examples exist.

Genetic modification of plants is being achieved by direct DNA transfer via vector-independent and vector-dependent means since the early 1980s. Vector-independent methods with protoplasts include electroporation (Potrykus *et al.*, 1985), liposome fusion (Deshayes *et al.*, 1985), and microinjection (Crossway *et al.*, 1986), as well as high-velocity microprojectile bombardment (biolistics) (Klein *et al.*, 1987). This latter method can be executed with cells, tissues, and organs. The use of *Agrobacterium* in vector-mediated transfer has progressed very rapidly since the first reports of stable transformation (DeBlock *et al.*, 1984; Horsch *et al.*, 1984). Although the early transformations utilized protoplasts, regenerable organs such as leaves, stems, and roots have been subsequently used (Gasser & Fraley, 1989; Uchimiya *et al.*, 1989). Much of the research activity utilizing these tools has focused on engineering important agricultural traits for the control of insects, weeds, and plant diseases.

Pathogen-Free Plants and Germplasm Storage

Although these two uses of *in vitro* technology may appear unrelated, a major use of pathogen-free plants is for germplasm storage and the movement of living material across international borders (Thorpe, 1990). The ability to rid plants of viruses, bacteria, and fungi by culturing meristem tips has been widely used since the 1960s. The approach is particularly needed for virus-infected material, as bactericidal and fungicidal agents can be used successfully in ridding plants of bacteria and fungi (Bhojwani & Razdan, 1983). Meristem-tip culture is often coupled with thermotherapy or chemotherapy for virus eradication (Kartha, 1981).

Traditionally, germplasm has been maintained as seed, but the ability to regenerate whole plants from somatic and gametic cells and shoot apices has led to their use for storage (Kartha, 1981; Bhojwani & Razdan, 1983). Three *in vitro* approaches have been developed, namely use of growth retarding compounds (e.g., maleic hydrazide, B995, and ABA; Dodds, 1989), low nonfreezing temperatures (1–9°C; Bhojwani & Razdan, 1983), and cryopreservation (Kartha, 1981). In this last approach, cell suspensions, shoot apices, asexual embryos, and young plantlets, after treatment with a cryoprotectant, are frozen and stored at the temperature of liquid nitrogen (ca. −196°C) (Kartha, 1981; Withers, 1985)

Clonal Propagation

The use of tissue culture technology for the vegetative propagation of plants is the most widely used application of the technology. It has been used with all classes of plants (Murashige, 1978; Conger, 1981), although some problems still need to be resolved, e.g., hyperhydricity and abberant plants. There are three ways by which micropropagation can be achieved. These are enhancing axillary bud-breaking, production of adventitious buds directly or indirectly via callus, and somatic embryogenesis directly or indirectly on explants (Murashige, 1974, 1978). Axillary bud-breaking produces the smallest number of plantlets, but they are generally genetically true-to-type, while somatic embryogenesis has the potential to produce the greatest number of plantlets but is induced in the lowest number of plant species. Commercially, numerous ornamentals are produced, mainly via axillary bud-breaking (Murashige, 1990). As well, there are lab-scale protocols for other classes of plants, including field and vegetable crops and fruit, plantation, and forest trees, but cost of production is often a limiting factor in their use commercially (Zimmerman, 1986).

Product Formation

Higher plants produce a large number of diverse organic chemicals, which are of pharmaceutical and industrial interest. The first attempt at the large-scale culture of plant cells for the production of pharmaceuticals took place in the 1950s at the

Charles Pfizer Company (U.S.). The failure of this effort limited research in this area in the U.S., but work in Germany and Japan, in particular, led to development so that by 1978 the industrial application of cell cultures was considered feasible (Zenk, 1978). Furthermore, by 1987 there were 30 cell culture systems that were better producers of secondary metabolites than the respective plants (Wink, 1987). Unfortunately, many of the economically important plant products are either not formed in sufficiently large quantities or not at all by plant cell cultures. Different approaches have been taken to enhance yields of secondary metabolites. These include cell-cloning and the repeated selection of high-yielding strains from heterogeneous cell populations (Zenk, 1978; Dougall, 1987) and by using ELISA and radioimmunoassay techniques (Kemp & Morgan, 1987). Another approach involves selection of mutant cell lines that overproduce the desired product (Widholm, (1987). As well, both abiotic factors, such as UV irridiation, exposure to heat or cold and salts of heavy metals, and biotic elicitors of plant and microbial origin, have been shown to enhance secondary product formation (Eilert, 1987; Kurz, 1988). Last, the use of immobilized cell technology has also been examined (Brodelius, 1985; Yeoman, 1987).

Central to the success of producing biologically active substances commercially is the capacity to grow cells on a large scale. This is being achieved using stirred tank reactor systems and a range of air-driven reactors (Fowler, 1987). For many systems, a two-stage (or two-phase) culture process has been tried (Beiderbeck & Knoop, 1987; Fowler, 1987). In the first stage, rapid cell growth and biomass accumulation are emphasized, while the second stage concentrates on product synthesis with minimal cell division or growth. However, by 1987 the naphthoquinone shikonin was the only commercially produced secondary metabolite from cell cultures (Fujita & Tabata, 1987).

THE PRESENT ERA

During the 1990s and the early twenty-first century continued expansion in the application of *in vitro* technologies to an increasing number of plant species has been observed. Tissue culture techniques are being used with all types of plants, including cereals and grasses (Vasil & Vasil, 1994), legumes (Davey *et al.,* 1994), vegetable crops (Reynolds, 1994), potato (Jones, 1994) and other root and tuber crops (Krikorian, 1994a), oilseeds (Palmer & Keller, 1994), temperate (Zimmerman & Swartz, 1994) and tropical (Grosser, 1994) fruits, plantation crops (Krikorian, 1994b), forest trees (Harry & Thorpe, 1994), and, of course, ornamentals (Debergh, 1994). As will be seen from these articles, the application of *in vitro* cell technology goes well beyond micropropagation and embraces all the *in vitro* approaches that are relevant or possible for the particular species and the problem(s) being addressed. However, only limited success has been achieved in exploiting somaclonal variation (Karp, 1994) or in the regeneration of useful plantlets from mutant cells (Dix, 1994); also, the early promise of protoplast technology remains largely unfulfilled (Feher & Dudits, 1994). Good

progress is being made in extending cryopreservation technology for germplasm storage (Kartha & Engelmann, 1994). Progress is also being made in artificial seed technology (Redenbaugh, 1993).

Cell cultures have remained an important tool in the study of plant biology. Thus progress is being made in cell biology, for example, in studies of the cytoskeleton (Kong *et al.*, 1998), on chromosomal changes in cultured cells (Kaeppler & Phillips, 1993), and in cell cycle studies (Komamine *et al.*, 1993; Trehin *et al.*, 1998). Better physiological and biochemical tools have allowed for a reexamination of neoplastic growth in cell cultures during habituation and hyperhydricity and relate it to possible cancerous growth in plants (Gaspar, 1995). Cell cultures have remained an extremely important tool in the study of primary metabolism; for example, the use of cell suspensions to develop *in vitro* transcription systems (Suguira, 1997) or the regulation of carbohydrate metabolism in transgenics (Stitt & Sonnewald, 1995). The development of medicinal plant cell culture techniques has led to the identification of more than 80 enzymes of alkaloid biosynthesis (reviewed in Kutchan, 1998). Similar information arising from the use of cell cultures for molecular and biochemical studies on other areas of secondary metabolism is generating research activity on metabolic engineering of plant secondary metabolite production (Verpoorte *et al.*, 1998).

Cell cultures remain an important tool in the study of morphogenesis, even though the present use of developmental mutants, particularly of *Arabidopsis*, is adding valuable information on plant development (e.g., see *The Plant Cell* (Special Issue), July, 1997). Molecular, physiological, and biochemical studies are allowing for in-depth understanding of cytodifferentiation, mainly tracheary element formation (Fukuda, 1997), organogenesis (Thorpe, 1993; Thompson & Thorpe, 1997), and somatic embryogenesis (Nomura & Komamine, 1995; Dudits *et al.*, 1995).

Advances in molecular biology allow for the genetic engineering of plants through the precise insertion of foreign genes from diverse biological systems. Three major breakthroughs have played major roles in the development of this transformation technology (Hinchee *et al.*, 1994). These are the development of shuttle vectors for harnessing the natural gene transfer capability of *Agrobacterium* (Fraley *et al.*, 1985), the methods to use these vectors for the direct transformation of regenerable explants obtained from plant organs (Horsch *et al.*, 1985), and the development of selectable markers (Cloutier & Landry, 1994). For species not amenable to *Agrobacterium*-mediated transformation, physical, chemical, and mechanical means are used to get the DNA into the cells. With these latter approaches, particularly biolistics, it is becoming possible to transform any plant species and genotype.

The initial wave of research in plant biotechnology has been driven mainly by the seed and agrichemical industries and has concentrated on "agronomic traits" of direct relevance to these industries, namely the control of insects, weeds, and plant diseases (Fraley, 1992). At present, over 100 species of plants have been genetically engineered, including nearly all the major dicotyledonous

crops and an increasing number of monocotyledonous ones as well as some woody plants. Current research has led to routine gene transfer systems for most important crops. In addition, technical improvements are further increasing transformation efficiency, extending transformation to elite commercial germplasm and lowering transgenic plant production costs. The next wave in agricultural biotechnology is already in progress with biotechnological applications of interest to the food processing, speciality chemical, and pharmaceutical industries. Also see Datta (2007).

The current emphasis and importance of plant biotechnology can be gleaned from the IXth International Congress on Plant Tissue and Cell Culture held in Israel in June, 1998. The theme of the Congress was "Plant Biotechnology and In Vitro Biology in the 21st Century." This theme was developed through a scientific program which focused on the most important developments, both basic and applied, in the areas of plant tissue culture and molecular biology and their impact on plant improvement and biotechnology (Thorpe & Lorz, 1998). The titles of the plenary lectures were (1) "Plant Biotechnology Achievements and Opportunities at the Threshold of the 21st Century," (2) "Towards Sustainable Crops via International Cooperation," (3) "Signal Pathways in Plant Disease Resistance," (4) "Pharmaceutical Foodstuffs: Oral Immunization with Transgenic Plants," (5) "Plant Biotechnology and Gene Manipulation," and (6) "Use of Plant Roots for Environmental Remediation and Chemical Manufacturing". These titles not only clearly show where tissue culture was but where it was heading, as an equal partner with molecular biology as a tool in basic plant biology and in various areas of application. Later Congresses in Florida, USA (2002), Beijing, China (2006), and St Louis, Missouri, USA (2010) supported this view and clearly demonstrated the advances that were being made in these areas. Also see Datta (2007) and Stasolla and Thorpe (2011). As Schell (1995) pointed out, progress in applied plant biotechnology is fully matching and is in fact stimulating fundamental scientific progress.

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